ELSEVIER

International Journal of Antimicrobial Agents 27 (2006) 138–151

INTERNATIONAL JOURNAL OF Antimicrobial Agents

www.ischemo.org

DNA microarray detection of antimicrobial resistance genes in diverse bacteria

Jonathan G. Frye [a,*], Troy Jesse [a], Fred Long [b], Gaelle Rondeau [b], Steffen Porwollik [b], Michael McClelland [b], Charlene R. Jackson [a], Mark Englen [a], Paula J. Fedorka-Cray [a]

[a] *Bacterial Epidemiology and Antimicrobial Resistance Research Unit, U.S. Department of Agriculture, Agriculture Research Service, Richard B. Russell Research Center, 950 College Station Road, Athens, GA 30605, USA*

[b] *Sidney Kimmel Cancer Center, 10835 Road to the Cure, San Diego, CA 92121, USA*

Received 10 August 2005; accepted 17 September 2005

Abstract

High throughput genotyping is essential for studying the spread of multiple antimicrobial resistance. A test oligonucleotide microarray designed to detect 94 antimicrobial resistance genes was constructed and successfully used to identify antimicrobial resistance genes in control strains. The microarray was then used to assay 51 distantly related bacteria, including Gram-negative and Gram-positive isolates, resulting in the identification of 61 different antimicrobial resistance genes in these bacteria. These results were consistent with their known gene content and resistance phenotypes. Microarray results were confirmed by polymerase chain reaction and Southern blot analysis. These results demonstrate that this approach could be used to construct a microarray to detect all sequenced antimicrobial resistance genes in nearly all bacteria.

Published by Elsevier B.V. on behalf of International Society of Chemotherapy

Keywords: Microarray; Antimicrobial resistance; Horizontal gene transfer

1. Introduction

Shortly after the introduction of antimicrobials to treat bacterial infections, resistance to these compounds was observed in the bacteria they initially controlled [1]. The development of antimicrobial resistance in bacteria renders some infections untreatable today and antimicrobial resistance is now a major health concern [2]. Although many different mechanisms are responsible for antimicrobial resistance, two main genetic events generate the majority of antimicrobial resistance currently observed: the mutation of native genes to resistant alleles; and the acquisition of foreign genes (either chromosomal or extrachromosomal) that confer a resistant phenotype [3]. Foreign genes have been the subject of intense investigation because they can be acquired horizontally via conjugation, transformation or transduction, and they may be transmitted in groups of genes on plasmids, transposons or integrons as often observed in multidrug-resistant (MDR) bacteria [4]. Currently, resistant bacterial phenotypes are characterised by growth in the presence of antimicrobials using test methodology such as Sensititer™ broth microdilution, Etest and disk diffusion [5,6]. Identification methods for the genes that cause resistance have typically been limited to techniques such as polymerase chain reaction (PCR) and Southern blotting, which can be cumbersome and can only detect one or a few genes at a time [7]. Therefore, identifying the genes responsible for resistance can require arduous screening for hundreds of possible antimicrobial resistance genes.

The mention of trade names or commercial products in this manuscript is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

* Corresponding author. Tel.: +1 706 546 3677; fax: +1 706 546 3172.
E-mail address: jfrye@saa.ars.usda.gov (J.G. Frye).

0924-8579/$ – see front matter. Published by Elsevier B.V. on behalf of International Society of Chemotherapy
doi:10.1016/j.ijantimicag.2005.09.021

With recent technical advances, it is now possible to detect thousands of genes simultaneously using DNA microarrays [8]. Several studies have reported the development of microarrays, including arrays for the detection of antimicrobial resistance genes, using PCR product probes [8–10]. However, construction of these microarrays is time consuming and requires a template for each gene of interest, design and synthesis of gene-specific primers, PCR synthesis, gel scoring, and purification of PCR products before a functional microarray can be constructed. These difficulties are avoided in a microarray incorporating synthetic oligonucleotide probes, an approach that has been frequently described for gene expression and comparative genomic hybridisations [11–14]. Recent studies have reported the design of oligonucleotide microarrays for the detection of antimicrobial resistance genes in either *Salmonella enterica* or Gram-positive bacteria [15,16]. However, the potential of horizontal gene exchange between distantly related bacteria necessitates the development of a common detection system to study the spread of antimicrobial resistance in all bacteria. This study presents the design and construction of a test DNA microarray for the detection of antimicrobial resistance genes in virtually any bacteria. The techniques described in this report enable efficient and inexpensive design and construction of customised oligonucleotide microarrays for the detection of multiple antimicrobial resistance genes regardless of the host bacteria. These methods can be used to construct a microarray to detect nearly all sequenced antimicrobial resistance genes simultaneously. This type of tool will be key to understanding the acquisition, transmission and dissemination of antimicrobial resistance in pathogenic and commensal bacteria.

2. Materials and methods

2.1. Oligonucleotide probe design

The sequences of 94 genes (Table 1) to be detected were obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). When possible, standard nomenclature (http://faculty.washington.edu/marilynr/) for these genes was applied [17–19]. In cases without generally agreed upon names, the name in the GenBank annotation corresponding to the sequence accession number was used. All possible 70-mer probes for all genes were analysed with the program MELTING [20] to determine their melting temperature (T_m). The average T_m for all probes was 72.74 °C. For each gene, the probe closest to the average T_m of all probes was selected for further analysis. The probe was discarded if it contained an 8-mer mononucleotide or dinucleotide repeat, or a self-annealing sequence. BLAT [21] analysis of the probes against the NCBI database was used to determine whether the probe was unique to the resistance gene(s) of interest. Probes that failed at any step were discarded and the selection process was repeated until a suitable oligonucleotide was designed for each gene. Although these diverse genes varied in their GC content, the range of T_m values for these probes was 67.83 °C to 76.68 °C, which was appropriate for the hybridisation conditions [22]. Probes were synthesised (Qiagen, Germantown, MD) with the addition of a universal linker (5′-CTAGATCGAC-3′), a C-6 spacer and an amino modification at the 5′ end. For quality control, 12 probes were synthesised in duplicate or triplicate and arrayed independently (indicated in Tables 1 and 2 by *).

2.2. Microarray construction

Oligonucleotide probes were dissolved in 50% dimethylsulphoxide at a concentration of 40 μM and spotted in triplicate onto Corning UltraGAPS slides (Corning Inc., Life Sciences, Acton, MA) with an Omnigrid robot (Genemachines, San Carlos, CA) and post processed as previously described for PCR product arrays [22]. Approximately 5000 PCR probes from a *S. enterica* microarray were also spotted onto the slide. The *Salmonella* microarray has been described previously [22,23] and covers 99.4% of the *S. enterica* serovar Typhimurium LT2 genome (4466 genes) and 98.3% of the *S. enterica* serovar Typhi CT18 genome (4521 genes). The PCR products are used as internal controls for the quality of hybridisations. The PCR probe for the 23S ribosomal RNA gene (*rrlH*) is an internal control for bacterial genomic DNA hybridisation.

2.3. Strains, growth conditions and antimicrobial susceptibility

The fully sequenced control strains were *S. enterica* serovar Typhimurium LT2 (*S.* Typhimurium LT2) [24] and *S. enterica* serovar Typhi CT18 (*S.* Typhi CT18) [25]. *Enterococcus* control strains obtained from the American Type Culture Collection (ATCC, Manassas, VA) are indicated by their ATCC numbers in Table 2. *Enterococcus faecium* 10N551023 [26], *Staphylococcus aureus* RN4220 [27] and *Streptococcus pyogenes* O2C1061 [28] have been previously described in their corresponding references. Test isolates of *Salmonella* serovars, *Escherichia coli*, *Enterococcus* spp. and *Campylobacter jejuni* were obtained from the National Antimicrobial Resistance Monitoring System (NARMS) bacterial collection. Phenotypic analysis was conducted as previously described (http://www.cdc.gov/narms/). Bacteria were grown from frozen stock cultures stored at −70 °C by standard methods with appropriate media. *Salmonella* and *E. coli* were grown in Luria–Bertani (LB) media, on LB agar or blood agar plates (BAPs) at 37 °C as indicated. *Campylobacter* were grown on Campy-Cefex plates and incubated at 42 °C for 48 h under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) in zip-top storage bags. *Enterococcus* spp. were grown in LB, brain–heart infusion (BHI) media or on BAPs. Susceptibility testing for *Salmonella*, *E. coli* and *Enterococcus* was performed using custom-made broth microdilution plates for

Table 1
Genes and corresponding probes used in construction of the antimicrobial resistance microarray

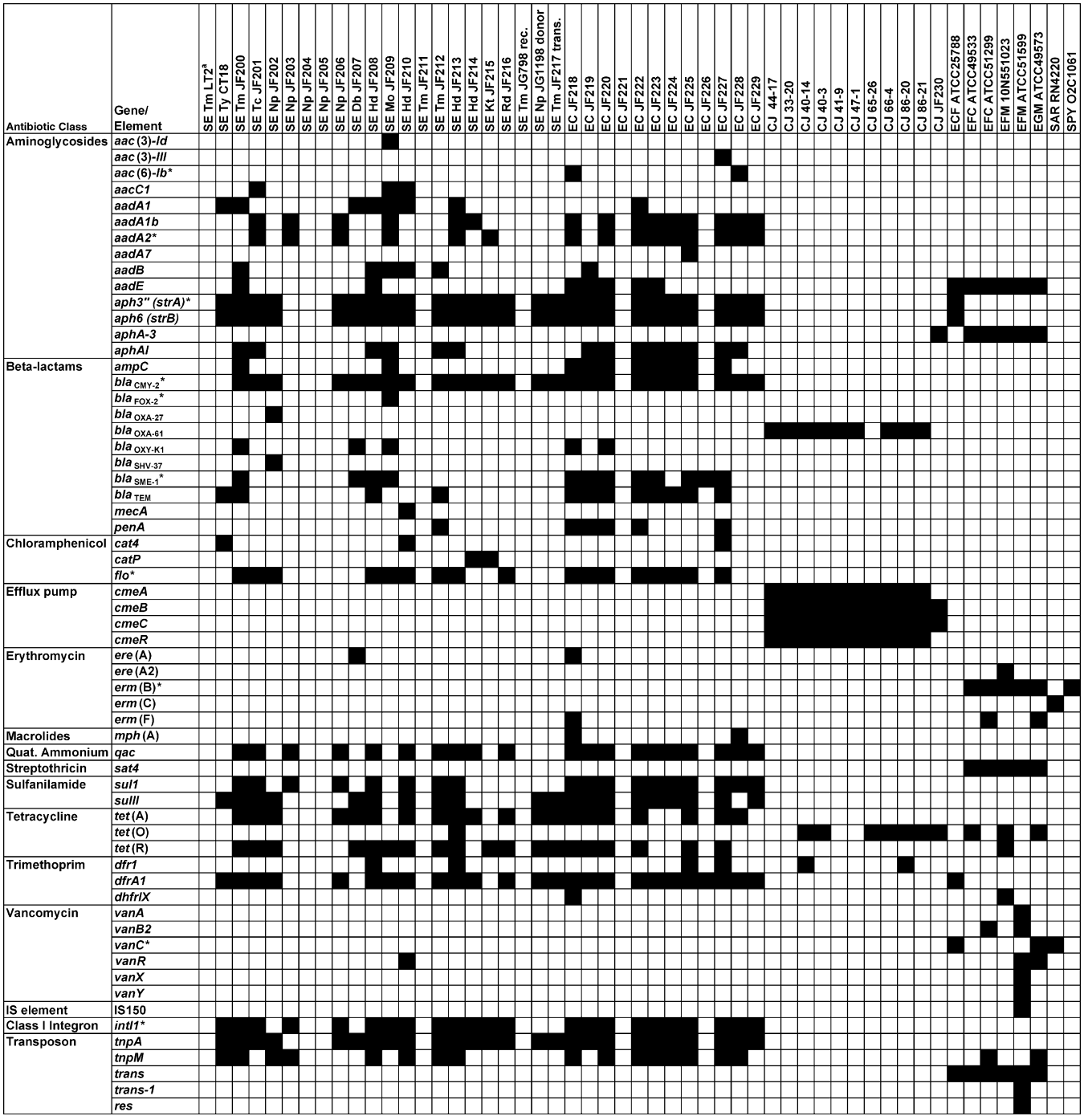
Gene/element[a]	Antibiotic class[b]	Oligonucleotide probe sequence (5'->3')[c]	GenBank Accession No.[d]	Location on array[e]
aac(3)-*Id*	**Aminoglycosides**	ATTAAAAAACTCAAGGCTATAGGCGCAGCGCGTGGAGCTTATGTGATTTACGTCCAAGCTGATAAAGGCG	**AY458224**	31
aac(3)-*III*	**Aminoglycosides**	CCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGG	**X13542**	34
aac(6)-*Ib**	**Aminoglycosides**	AAGTCGCCTGGAAAACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAA	**AY103455**	8, 16, 37
aacC1	**Aminoglycosides**	CCGCAGTGGCTCTCTATACAAAGTTGGGCATACGGGAAGAAGTGATGCACTTTGATATCGACCCAAGTAC	**U04610**	38
aadA1	**Aminoglycosides**	TCATATCGTTTTAACCCTGGCGCGTATCTGGTACACCCTTTCTACCGGGAGATTTACCTCTAAGGATGCG	**NC003198**	29
aadA1b	**Aminoglycosides**	AGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGC	**AJ62853**	18
*aadA2**	**Aminoglycosides**	AAAACGCCTACCTGCCCAGTATCAGCCCGTCTTACTTGAAGCTAAGCAAGCTTATCTGGGACAAAAAGAA	**AY263741**	2, 6
aadA7	**Aminoglycosides**	GATCTCTTCAGCTCAGTCCCAGAAAGCGATCTATTCAAGGCACTGGCCGATACTCTGAAGCTATGGAACT	**AY458224**	4
aadB	**Aminoglycosides**	TACTTTTACTATGCCGATGAAGTACCACCAGTGGACTGGCCTACAAAGCACATAGAGTCCTACAGGCTCG	**AY204504**	11
aadE	**Aminoglycosides**	GAAGCATTATTTCTATGCCATCAATTGTTCAGGGCGGTATCCGGTGAGGTGGCGGAAAGGCTTCATTATG	**AF516335**	47
aph	**Aminoglycosides**	TTCTTGAGCTTCTCGGGCAGACGGAACTAACCGTCAACAAAATCGGATATTCCGGAGATCACGTCTATCA	**X03364**	44
*aph3" (strA)**	**Aminoglycosides**	TTTTTGGTGAATCGCATTCTGACTGGTTGCCTGTCAGAGGCGGAGAATCTGGTGATTTTGTTTTTCGACG	**AY055428**	15, 28
aph6 (strB)	**Aminoglycosides**	TCATTGCCAGACGGGACTCCTGCAATCGTCAAGGGATTGAAACCTATAGAAGACATTGCTGATGAACTGC	**AY055428**	5
aphA-3	**Aminoglycosides**	CAGGCTCTTTCACTCCATCGACATATCGGATTGTCCCTATACGAATAGCTTAGACAGCCGCTTAGCCGAA	**AF516335**	59
aphAI	**Aminoglycosides**	TGCTCGAGGCCGCGATTAAATTCCAACCTGGATGCTGATTTATATGGGTATAGATGGGCTCGCGATAATG	**U13633**	20
ampC	**Beta-lactams**	CACTATTTGAGCTCGGATCTGTAAGTAAAACTTTCACAGGTGTGCTGGGTGCGGTTTCTGTGGCGAAAAA	**AJ237702**	83
ampR	**Beta-lactams**	CTGGCCGGATTCTATGACAGCCATCCGCATATTGATCTGCATATCTCCACCCATAACAATCATGTGGACC	**AJ237702**	66
bla$_{CMY-2}$*	**Beta-lactams**	ATATCGCCAATAACCACCCAGTCACGCAGCAAACGCTGTTTGAGCTAGGATCGGTTAGTAAGACGTTTAA	**X91840**	1, 78
bla$_{CTX-M-12}$	**Beta-lactams**	AGACTGGGTGTGGCATTGATTAACACAGCGGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTCG	**AF305837**	80
bla$_{FOX-2}$*	**Beta-lactams**	AATGACAAGATGCAAACTTACTATCGGAGCTGGTCACCGGTTTATCCGGCGGGGACCCATCGCCAGTATT	**Y10282**	68, 72
bla$_{IMP-2}$	**Beta-lactams**	ATTGGTTTGTGGAGCGCGGCTATAAAATCAAAGGCACTATTTCCTCACATTTCCATAGCGACAGCACAGG	**AJ243491**	65
bla$_{KPC-3}$	**Beta-lactams**	CATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATCTCGGAAAAATATCTGACAACAGGCATG	**AF395881**	64
bla$_{OXA-2}$	**Beta-lactams**	GCAGGCCACAATCAAGACCAAGATTTGCGATCAGCAATGCGGAATTCTACTGTTTGGGTGTATGAGCTAT	**M95287**	24
bla$_{OXA-26}$	**Beta-lactams**	GTTACTCCACAGGTAGGTTGGTTGACTGGTTGGGTGGAGCAAGCTAATGGAAAAAAAATCCCCTTTTCGC	**AF201287**	62
bla$_{OXA-27}$	**Beta-lactams**	GGCGAGAAAAGGTCATTTACCGCTTGGGAAAAAGACATGACACTAGGAGAAGCCATGAAGCTTTCTGCAG	**AF201828**	61
bla$_{OXA-2b}$	**Beta-lactams**	AGCAATAAAGAGGTGGTAAATAAAAGGCTGGAGATTAACGCAGCCGATTTGGTGGTCTGGAGCCCGATTA	**AY303807**	63
bla$_{OXA-61}$	**Beta-lactams**	ATGATGGAAAAACTTGGGCGAGTAACGACTTTTCAAGGGCTATGGAGACTTTCTCTCCCGCTTCCACTTT	**AY587956**	108
bla$_{OXA-9}$	**Beta-lactams**	TCCGTGCTCGTCTTTTAAACTTCCATTGGCAATCATGGGGTTTGATAGTGGAATCTTGCAGTCGCCAAAA	**M55547**	49
bla$_{OXY-K1}$	**Beta-lactams**	GCACCACCAATGATATTGCGGTTATCTGGCCGGAAGATCACGCTCCGCTGATATTAGTCACCTACTTTAC	**AF473577**	69
bla$_{PER-2}$	**Beta-lactams**	TTATGGAAATGGATGGTTGAAACCACCACAGGACCACAGCGGTTAAAAGGCTTGTTACCTGCTGGTACTA	**X93314**	75
bla$_{PSE-1}$	**Beta-lactams**	TGTGGAGTGAGCATCAAGCCCCAATTATTGTGAGCATCTATCTAGCTCAAACACAGGCTTCAATGGCAGA	**AB126603**	12
bla$_{ROB-1}$	**Beta-lactams**	TATTATTGCTGACATTAACGGCTTGTTCGCCCAATTCTGTTCATTCGGTAACGTCTAATCCGCAGCCTGC	**AF022114**	81
bla$_{SHV-37}$	**Beta-lactams**	CTTGAGCAAATTAAACTAAGCGAAAGCCAGCTGTCGGGCCGCGTAGGCATGATAGAAATGGATCTGGCCA	**AF317502**	55, 74
bla$_{SME-1}$*	**Beta-lactams**	GATGAGCGGTTCCCTTTATGCAGTTCATTTAAAGGTTTTTTGGCGGCTGCTGTTTTAGAGAGGGTGCAAC	**Z28968**	70, 76
bla$_{SME-2}$*	**Beta-lactams**	TTAGGTTAGATCGCTGGGAACTGGAACTTAACACTGCAATCCCAGGAGATAAACGTGACACTTCAACGCC	**AF275256**	82
bla$_{TEM}$	**Beta-lactams**	TTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGAT	**AJ634602**	21
bla$_{VIM-2}$	**Beta-lactams**	TTATTGGTCTATTTGACCGCGTCTATCATGGCTATTGCGAGTCCGCTCGCTTTTTCCGTAGATTCTAGCG	**AF369871**	77
mecA	**Beta-lactams**	AATTGGCAAATCCGGTACTGCAGAACTCAAAATGAAACAAGGAGAAACTGGCAGACAAATTGGGTGGTTT	**AY271717**	42
mecI	**Beta-lactams**	TGCAAGTGCGAATAATATAATAGAAGAAATACAAATGCAAAAGGACTGGAGTCCAAAAACCATTCGTACA	**D86934**	54
penA	**Beta-lactams**	ACTTATCCGACGTTGGATGGCGAGAACGTCTTATAGTGACTCTGTCAACTGCCACACTCAACCTATCTCG	**L02928**	84
ble	**Bleomycin**	ATGGATTCGCAGTTCTAATGTGTAATGAGGTTCGGATTCATCTATGGGAGGCAAGTGATGAAGGCTGGCG	**D86934**	60
cat	**Chloramphenicol**	CGACATGAAGAGTTCAGGACCGCATTAGATGAAAACGGACAGGTAGGCGTTTTTTCAGAAATGCTGCCTT	**M35190**	91
cat4	**Chloramphenicol**	CCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGAAATTCCGTATGGCAATGAAAGACGGTGA	**AJ401050**	43
catP	**Chloramphenicol**	TGGCAATTCAAGTTCATCACGCAGTATGTGACGGATTTCACATTTGCCGTTTTGTAAACGAATTGCAGGA	**L02937**	13
*flo**	**Chloramphenicol**	GATATTCATTACTTTGGCTATACTGGCGATGCTCGCACTCCTAAATGCGGGTTTCAGGTGGCACGAAACC	**AF252885**	32, 37,73
cmeA	**Efflux pump**	TTTAGGTGTTGTGCTTTTACTCGCTGCTTGCAGCAAAGAAGAAGCACCAAAAATACAAATGCCGCCTCAA	**AR466820**	103
cmeB	**Efflux pump**	TTGCCAATGATTTTCGCAACAGGAGCAGGAAGTGCTTCAAGACACTCTTTAGGAACAGGGCTTATTGGTG	**AR466820**	109
cmeC	**Efflux pump**	AAAATTTGATGGTAGCGCAAGCGGAAGTCGTGCAAAAACAGCTATAAATGCTCCAAGCAATCGAACTGGG	**AR466820**	7
cmeR	**Efflux pump**	ACTCAAATAGAACACCATCACAAAAAGTTTTAGCCAGACAAGAAAAAATCAAAGCAGTGGCCTTAGAGCT	**AR466820**	97
mef(A) *mef*(E)*	**Efflux pump**	CGATTTTGGGACCTGCCATTGGTGTGCTAGTGGATCGTCATGATAGGAAGAAGATAATGATTGGTGCCGA	**AY319932**	94,100
msr(A)	**Efflux pump**	CACCACGGAAATCGCTAACGCCACACCGTTTTATTATGCCGAAGATGACCACCAGCAATATCTGCATAAA	**NC_002655**	101
vanA	**Glycopeptides**	AAAACCTTGCGCGGAATGGGAAAACGACAATTGCTATTCAGCTGTACTCTCGCCGGATAAAAAAATGCAC	**AF516335**	45

Table 1 (*Continued*)

Gene/element[a]	Antibiotic class[b]	Oligonucleotide probe sequence (5'->3')[c]	GenBank Accession No.[d]	Location on array[e]
vanB2	**Glycopeptides**	AAAAGTCGCAATCATCTTCGGCGGTTGCTCGGAGGAACATGATGTGTCGGTAAAATCCGCAATAGAAATT	**AY145441**	79
*vanC**	**Glycopeptides**	CACCGTTTCTTTAGCTTCAGCAACTAGCGCAATCGAAGCACTCCAATCATCTCCCTATGACTACGACCTC	**L29638**	85, 86
vanD	**Glycopeptides**	TTTACTTCCTACAGCCGTTATCCCCGCATGATGACAGCAGCCGGTTTTACGCTTACTGAAATACTGGATC	**AY489045**	92
vanE	**Glycopeptides**	TGGTTGTGGTATTTTAGGAAATGAACAATTGGTCGTTGGAGAATGTGACCAAATCAGTCTTGTGGATGGC	**AF430807**	98
vanG	**Glycopeptides**	AATTGGCAGGAATACCTGTTGTTGGCTGCGATACACTCTCATCAGCTCTTTGTATGGATAAGGACAGGGC	**AF253562**	104
vanH	**Glycopeptides**	GAATCCAACGCCAAATCCGCGCCTTTCAATCAATGTATCAGTGTGGGACATAAATCAGAGATTTCCGCCT	**AF516335**	39
vanR	**Glycopeptides**	ATCATGCTTCCCGGCACAAGCGGCCTTACTATCTGTCAAAAAATAAGGGACAAGCACACCTATCCGATTA	**AF516335**	56
vanX	**Glycopeptides**	TACCGTCCTAATCGTGCTGTAAACTGTTTTATGCAATGGGCTGCACAGCCGGAAAATAACCTGACAAAGG	**AF516335**	51
vanY	**Glycopeptides**	TTGATGAGCAAAGTGTGCTTTACCAAGAAATGGGGGCTGAGTATGCCTTACCAGCAGGTTATAGTGAGCA	**AF516335**	57
linB	**Lincosamide**	TGAAACATAGTATAACCTCGAACTTTGATTCATCCAACTGGTTGTTTGACGTAGCTCCGTACTTGATGCT	**AJ238249**	102
ere (A)	**Macrolides**	CGCAATTGGCCGAAATTATCCAGCTCATCGATCACCTCATGAAACCGCACGTTGATATGTTGACTCACTT	**A15069**	106
ere (A2)	**Macrolides**	CCGGTGCTCATGAACTTGAGCGATTTTCGGATACCCTGACCTTTTCTTTGTATGGCTCAGTGCTGATTTG	**AF512546**	25
ere (B)	**Macrolides**	GCAGGGCGATATGGGTGCAAAAGACAAATACATGGCAGATTCTGTGCTGTGGCATTTAAAAAACCCACAA	**A15097**	89
erm (A)	**Macrolides**	AAGTGGGTAAACCGTGAATATCGTGTTCTTTTCACTAAAAACCAATTCCGACAGGCTTTGAAGCATGCAA	**D86934**	48
erm (B)*	**Macrolides**	ACAAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATT	**AJ243541**	46, 93
erm (C)	**Macrolides**	TTTGAAATCGGCTCAGGAAAAGGCCATTTTACCCTTGAATTAGTAAAGAGGTGTAATTTCGTAACTGCCA	**NC001386**	99
erm (F)	**Macrolides**	GATTTGAAACTTGTCTATGAGGTAGGTCCTGAAAGTTTCTTGCCACCGCCAACTGTCAAATCAGCCCTGT	**U30830**	105
erm (G)	**Macrolides**	TTTGAAATAGGTGCAGGGAAAGGTCATTTTACTGCTGAATTGGTAAAGAGATGTAATTTTGTTACGGCGA	**M15332**	88
erm (TR)	**Macrolides**	AGAGGGGATTTGCTAAAAGGTTGCAAAATACCCAACGAGCTTTAGGTTTGCTGTTAATGGTGGAAATGGA	**AF002716**	87
mph (A)	**Macrolides**	CCGACATGGGCTCAAGCTCCATGGCCCGCTGACTGTCAATGAGCTTGGGCTCGACTATAGGATCGTGATC	**AY522923**	95
qac	**Quaternary ammonium**	GCAATAGTTGGCGAAGTAATCGCAACATCCGCATTAAAATCTAGCGAGGGCTTTACTAAGCTTGCCCCTT	**AY458224**	14
arr-3	**Rifampicin**	ATAATTACAAGCAGGTGCAAGGACCGTTCTATCATGGAACCAAAGCCAATTTGGCGATTGGTGACTTGCT	**AY038837**	71
sat (G) *vat* (E-8)	**Streptogramin**	ACTGACTGATTTGCCGTTGAAAGGTGATACTGTAGTCGGAAATGACGTGTGGTTTGGGCAAAATGTGACC	**AY043213**	90
vat (B)	**Streptogramin**	TGTGTGGATTGGTCAGAATGTTACTGTTATGCCAGGAATTCAAATAGGAGATGGAGCAATTGTTGCTGCG	**U19459**	107
vga (A)	**Streptogramin**	CGGGTACAATTGAAGGACGGGTATTGTGGAAAGCAAAAAGTTTTAGTATTCGCGGAGGAGACAAGATGGC	**M90056**	96
sat4	**Streptothricin**	TTGGAACCGGTACGCTTATATAGAAGATATCGCCGTATGTAAGGATTTCAGGGGGCAAGGCATAGGCAGC	**AF516335**	53
sul1	**Sulfanilamide**	CTACCTGAACGATATCCAAGGATTTCCTGACCCTGCGCTCTATCCCGATATTGCTGAGGCGGACTGCAGG	**AY458224**	23
sulII	**Sulfanilamide**	GAATAAATCGCTCATCATTTTCGGCATCGTCAACATAACCTCGGACAGTTTCTCCGATGGAGGCCGGTAT	**NC005324**	33
tet (A)	**Tetracycline**	CAGCCTGACCTCGATCGTCGGACCCCTCCTCTTCACGGCGATCTATGCGGCTTCTATAACAACGTGGAAC	**AJ634602**	30
tet (O)	**Tetracycline**	GAAAAGCAGAATATACCATCCACATAGAAGTCCCGCCAAATCCTTTCTGGGCTTCTGTCGGGTTGTCCAT	**M18896**	17
tet (R)	**Tetracycline**	ATGTTTATCAGTGATAAAGTGTCAAGCATGACAAAGTTGCAGCCGAATACAGTGATCCGTGCCGCCCTGG	**AJ634602**	3
dfr1	**Trimethoprim**	AGCCGGAAGGTGATGTTTACTTTCCTGAAATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGC	**AJ400733**	35
dfrA1	**Trimethoprim**	TTATCTCTCCTCCCGTCGTAACAGCAAAGCTGCATACCGGTTTCTGGGTAAAATCCTCAACAACGTGAAG	**AJ628353**	9
dhfrIX	**Trimethoprim**	CAGTTTTGATTATGGGTAGAAAAACTTTTGCCTCACTGCCTAAAGTGCTGCCCGGACGACTTCATGTGGT	**X57730**	26
*intI1**	**Class I Integron**	CTACTTGCATTACAGTTTACGAACCGAACAGGCTTATGTCAACTGGGTTCGTGCCTTCATCCGTTTCCAC	**AY458224**	22, 36
IS1182	**Insertion element**	CGGGCCGCCAAATACAAAATATGCTGATAGATAGTATTCGGATGCGCTGCTTATCTCAAGAGCAATTCCC	**AF516335**	41
IS150	**Insertion element**	AAAAGGGAACTGAGGGTTCACTGATTCTACATTCAGATCAAGGATGGCAGTATCAGATGCCACAATATCA	**AF516335**	58
res	**Transposon**	AATGGCTGGTGTTAACCAATTAGAGCGAGATCTTATTCGGATGAGACAACGTGAAGGGATTGAATTGGCT	**AF516335**	50
tnpA	**Transposon**	GATGATCAAAACGCAGGTTGTCAAACTGACTACGTTCACGCGGCGATTATAGCCGATCAAATGATGAGCA	**AJ628353**	19
tnpM	**Transposon**	CCAATGGAGGAACACCACCATGAACGCCAATGAACCGAGCACCAGTTGCTGCGTGTGCTGCAAGGAAATC	**AJ628353**	27
trans	**Transposon**	CGGGACACACAAGCAGCCTATGCTTTTCTTAAGCGGTTAGTGAAGCAGTTTGATGAACCGAAGGTTGTAG	**AF516335**	40
trans-1	**Transposon**	GAAGGCGGTGCTTCTTCACTTGAGAGCCAAAAAAGGGGCAGAAAAATTAGTATGAATTCCAAGCTAAACA	**AF516335**	52
(-) control		CTAGATCGACCTAGATCGACCTAGATCGACCTAGATCGACCTAGATCGACCTAGATCGAC		10

[a] Antimicrobial resistance genes are named according to the corresponding GenBank accession number; genes may have multiple names. * Indicates genes with multiple identical probes.
[b] Antimicrobial resistance classes are those described in GenBank annotations.
[c] Oligonucleotide probes are the coding (sense) strand of the genes.
[d] Accession numbers from GenBank are for one annotation of each gene; multiple accession numbers and annotations may exist for some genes.
[e] Probe location numbers correspond to the position on the microarray and to the numbers in Fig. 2.

Table 2
Genotypes of isolates as detected by microarray hybridisation



Positive hybridisations are indicated by a filled block (■); negative hybridisations are indicated by an open block (□).

[a]Isolates: SE, *Salmonella enterica*; Tm, serovar Typhimurium; Tc, serovar Typhimurium variant Copenhagen; Np, serovar Newport; Mo, serovar Montevideo; Ty, serovar Typhi; Db, serovar Derby; Hd, serovar Heidelberg; Kt, serovar Kentucky; Rd, serovar Reading; EC, *Escherichia coli*; CJ, *Campylobacter jejuni*; ECF, *Enterococcus casseliflavus*; EFC, *Enterococcus faecalis*; EFM, *Enterococcus faecium*; EGM, *Enterococcus gallinarum*; SAR, *Staphylococcus aureus*; SPY, *Streptococcus pyogenes*. Isolates used for the conjugation: recipient (SE Tm JG798 rec.), donor (SE Np JG1198 donor) and transconjugant (SE Tm JF217 trans.) are indicated.

*Indicates genes with duplicate or triplicate probes.

the Sensititer™ system (TREK Diagnostic Systems, Inc., Westlake, OH). Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) guidelines for interpretation and recommended quality control organisms were used. *Campylobacter* isolates were tested following CLSI guidelines using the Etest (AB Biodisk, Piscataway, NJ) [5,6].

2.4. DNA extraction and labelling

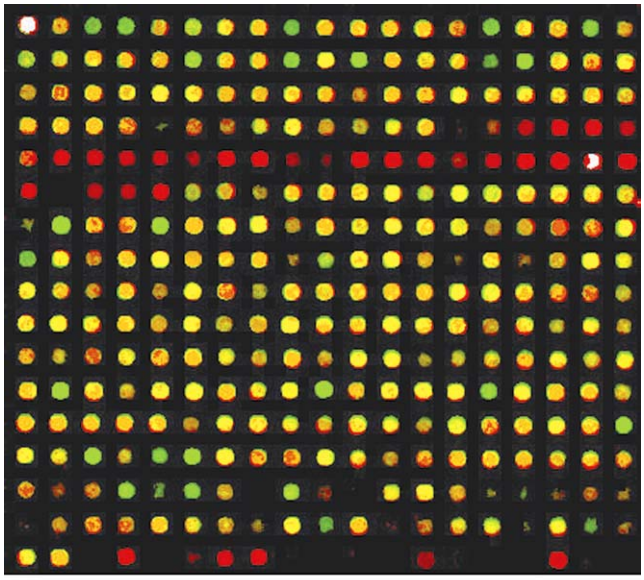
Genomic DNA from *Salmonella*, *E. coli* and Gram-positive bacteria was extracted from 5 mL of overnight cultures grown in LB (Gram-negative bacteria) or BHI (Gram-positive bacteria) media using the GenElute Bacterial Genomic DNA kit (Sigma, St Louis, MO) following specific instructions for Gram-negative and Gram-positive bacteria, respectively. *Campylobacter* genomic DNA was isolated from colonies collected from Campy-Cefex plates using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's directions. DNA was labelled with Cye dye-labelled dCTP (Amersham, Piscataway, NJ) via random priming and extension with Klenow fragment (New England Biolabs, Beverly, MA), followed by purification with a Qiagen PCR clean-up kit (Qiagen, Valencia, CA) as previously described [29]. DNA from experimental strains was labelled with Cy3, and control DNA from *S.* Typhimurium LT2 was labelled with Cy5.

2.5. Hybridisation and scanning

Dye-labelled DNA was dried and re-suspended in 80 μL of hybridisation buffer (25% formamide, 5× SSC, 0.1% sodium dodecyl sulphate, 1% bovine serum albumin), boiled for 5 min and applied to the microarray under a LifterSlip (Erie Scientific, Portsmouth, NH). Hybridisation was performed overnight in a hybridisation chamber (Corning Inc., Life Sciences, Acton, MA) submerged in a 42 °C water-bath. Protocols suggested by the manufacturer for hybridisations in formamide buffer were used for pre-hybridisation, hybridisation and post-hybridisation wash processes. Microarrays were scanned with a ScanArray Lite Laser scanner (Packard BioChip Technologies, Billerica, MA) using ScanArray Express 1.1 software.

2.6. Data analysis

Images were analysed and quantified using QUANTARRAY 3.0 software (Packard BioScience). Hybridisation signal intensities were measured by adaptive quantification followed by local background subtraction, and the medians of the triplicate spots were recorded. The control strain hybridisations were used to evaluate three techniques for interpreting the quantitative data: (1) an arbitrary cut-off of 2000 intensity units (IU); (2) a cut-off of two times the median of hybridisation intensity to all 70-mer probes (1556 IU in Fig. 1); and (3) a cut-off of the negative control plus two standard deviations (1209 IU in Fig. 1). Each interpretation yielded similar results. Therefore, an arbitrary cut-off of a median intensity above 2000 IU was reported as a positive hybridisation (Table 2) and intensities below 2000 IU were reported as negative.



	1	2	3	4	5	6	7	8	9	10
Gene	***bla*CMY-2**	***aadA2***	***tet*(R)**	***aadA7***	***aph6***	***aadA2***	***cmeC***	***aac(6)Ib***	***dfrA1***	**neg. con.**
Median Intesity	776	844	1091	665	2280	893	665	600	3951	937
Spot Score					+				+	

Fig. 1. Hybridisation results for the *Salmonella* genome antimicrobial resistance gene composite microarray. A single subarray from the 16 that make up the microarray is shown. The image is an overlay of hybridisation images of *Salmonella enterica* serovar Typhimurium LT2 (green) and serovar Typhi CT18 (red). LT2-specific gene probes are green, CT18-specific gene probes are red, and common gene probes appear yellow/orange. The ten oligonucleotide antimicrobial resistance gene probes on the bottom row are numbered as in Table 1. Background corrected and normalised intensity units (IU) for CT18 hybridisation to the antimicrobial resistance gene probes are indicated. The median hybridisation to all probes was 778 IU and the standard deviation of the negative control was 136 IU. Hybridisations were scored positive for antimicrobial resistance gene probes 5 *aph6* and 9 *dfrA1*.

2.7. Transfer of resistance genes by conjugation

Conjugations were carried out as previously described [30]. The donor strain was *S. enterica* serovar Newport JG1198, a strain harbouring a plasmid with the $bla_{\text{CMY-2}}$ gene conferring ampicillin resistance. The recipient strain was *S. enterica* serovar Typhimurium JG798, which was only resistant to nalidixic acid. Transconjugants were selected on LB agar containing 100 μg/mL ampicillin and 20 μg/mL nalidixic acid for counterselection.

2.8. PCR and Southern blotting

PCR reactions were performed as previously described [30–32]. Briefly, 100 ng of purified DNA was used as tem-

plate; products were separated by 1% agarose gel electrophoresis in 1× TBE buffer and visualised with ethidium bromide and ultraviolet transillumination. Presence of a product of the appropriate size was verified visually by comparison with positive controls. Southern blots were performed on purified genomic and plasmid DNA with a TurboBlotter (Schleicher & Schuell BioScience, Inc., Keene, NH) as previously described [30,33]. Labelled bla_{CMY-2} probes for the Southern blot analysis were prepared by PCR amplification using a Genius DIG labelling kit (Roche, Indianapolis, IN) and visualised with anti-DIG antibody and BCIP-NBT reagents.

3. Results

3.1. Design of oligonucleotide probes and validation of the antimicrobial resistance gene microarray construction

To test the application of oligonucleotide probes for the detection of sequenced antimicrobial resistance genes, 94 genes and genetic elements associated with antimicrobial resistance were chosen for probe design (Table 1). These were selected from a list of genes reported in *E. coli*, *S. enterica*, *Campylobacter* spp. and *Enterococcus* spp. as well as in other bacteria (reviewed in [34–40]). Genetic elements were chosen from the published literature to represent a wide range of antimicrobial resistance genes. The protocol described in Section 2.1 was used to design the oligonucleotide probes for microarray construction.

The oligonucleotides designed for the detection of antimicrobial resistance genes were synthesised and spotted onto a microarray of *S. enterica* PCR products that had been constructed from two sequenced strains [22]. This approach allowed the 70-mer probes to be tested in the context of a working microarray and compared directly with PCR probes ranging in size from 45 bp to 6108 bp with an average size of 866 bp. Duplicate or triplicate probes for 12 genes were independently synthesised and arrayed for quality control (indicated by * in Table 1). After printing and post processing using standard methods for the PCR product microarrays (Corning), the arrays were evaluated with Sybrgreen II® staining (Molecular Probes, Eugene, OR) [41]. Analysis of the array images showed that 70-mer probes treated as recommended for PCR products produced spots with comparable morphology and amount of DNA as spots produced from PCR products (data not shown).

3.2. Antimicrobial resistance gene microarray hybridisation to control strains

Initial test hybridisations were performed using two *S. enterica* strains with published sequences, serovar Typhimurium strain LT2 (antimicrobial sensitive) and serovar Typhi strain CT18 (multidrug resistant) [24,25]. Fig. 1 shows a hybridisation of LT2 and CT18 DNA to one of the 16 subarrays that make up this composite *Salmonella* antimicrobial resistance gene microarray. Labelled LT2 DNA hybridised to LT2-specific PCR probes (green) and to probes in common with CT18 (yellow/orange). Similarly, labelled CT18 DNA hybridised to CT18-specific (red) and LT2 shared PCR probes (yellow/orange). As expected, DNA from the sensitive LT2 strain did not hybridise to any of the 70-mer antimicrobial resistance gene probes. However, CT18 DNA hybridised with 12 antimicrobial resistance gene probes on the microarray, two of which, *aph6* (*strB*) and *dfrA1*, are on the subarray shown in Fig. 1. Quantitative image analysis showed that local background was less than 300 IU and the non-specific hybridisation to the 70-mer probes was less than 1000 IU, which were similar to those observed for PCR probes, indicating that 70-mer probes performed as well as PCR probes under these conditions.

Fig. 2 shows the staining of all oligonucleotides present in the antimicrobial resistance microarray with Sybrgreen II® (Fig. 2A) and the result of their hybridisation with the labelled CT18 DNA (Fig. 2B). Hybridisation intensities above the 2000 IU cut-off were scored as positive and were confirmed by visual inspection of the image. Positive hybridisations were detected to probes 5, 9, 15, 19, 21, 22, 27, 28, 29, 33, 36 and 43 corresponding to genes *aph6* (*strB*), *dfrA1*, *aph3*" (*strA*)*, *tnpA*, bla_{TEM}, *intI1**, *tnpM*, *aph3*" (*strA*)*, *aadA1*, *sulII*, *intI1** and *cat4*, respectively (* indicates duplicate probes). The results of quantitative analysis of the *S.* Typhi CT18 hybridisation are shown in Table 2, with detected genes indicated by filled blocks. Seven antimicrobial resistance genes were detected, including *aadA1* (aminoglycoside), *cat4* (chloramphenicol), *dfrA1* (trimethoprim), *aph3*" (*strA*) and *aph6* (*strB*) (streptomycin), *sulII* (sulfonamide) and bla_{TEM} (ampicillin). In addition, three mobile element genes were also detected: *tnpA* (Tn21 transposon), *tnpM* (Tn21 transposon) and *intI1* (Class I integrase). Comparison of the sequence of the probes with the published CT18 sequence showed that all of the probes perfectly matched the genes they detected (70/70 nt) except for *cat4*, *tnpA* and *intI1*, which had a single mismatch (69/70 nt) [25]. The microarray results confirmed the presence of the antimicrobial resistance genes previously identified on the CT18 pHCM1 plasmid by sequence analysis [25]. These genes were previously designated as *dhfr1b* (trimethoprim), *sulII* (sulfonamide), *catI* (chloramphenicol), *bla* (TEM-1; ampicillin) and *strAB* (streptomycin) in several studies of pHCM1 [34,42]. The microarray results were also consistent with the MDR phenotype previously reported for CT18 [34,42].

Oligonucleotide probe 18 for *aadA1b* hybridised at a low level to the CT18 DNA (Fig. 2). Sequence comparison of this probe with the related CT18 *aadA1* gene revealed 17 contiguous identical nucleotides. However, this gene was scored as not present due to a hybridisation intensity of 1367 IU, which was less than the threshold of 2000 IU. As expected, the *tet*(A) and *tet*(R) genes on pHCM1 were not detected by the microarray since the *tet*(AR) alleles chosen for microarray probe design were divergent from those found on pHCM1

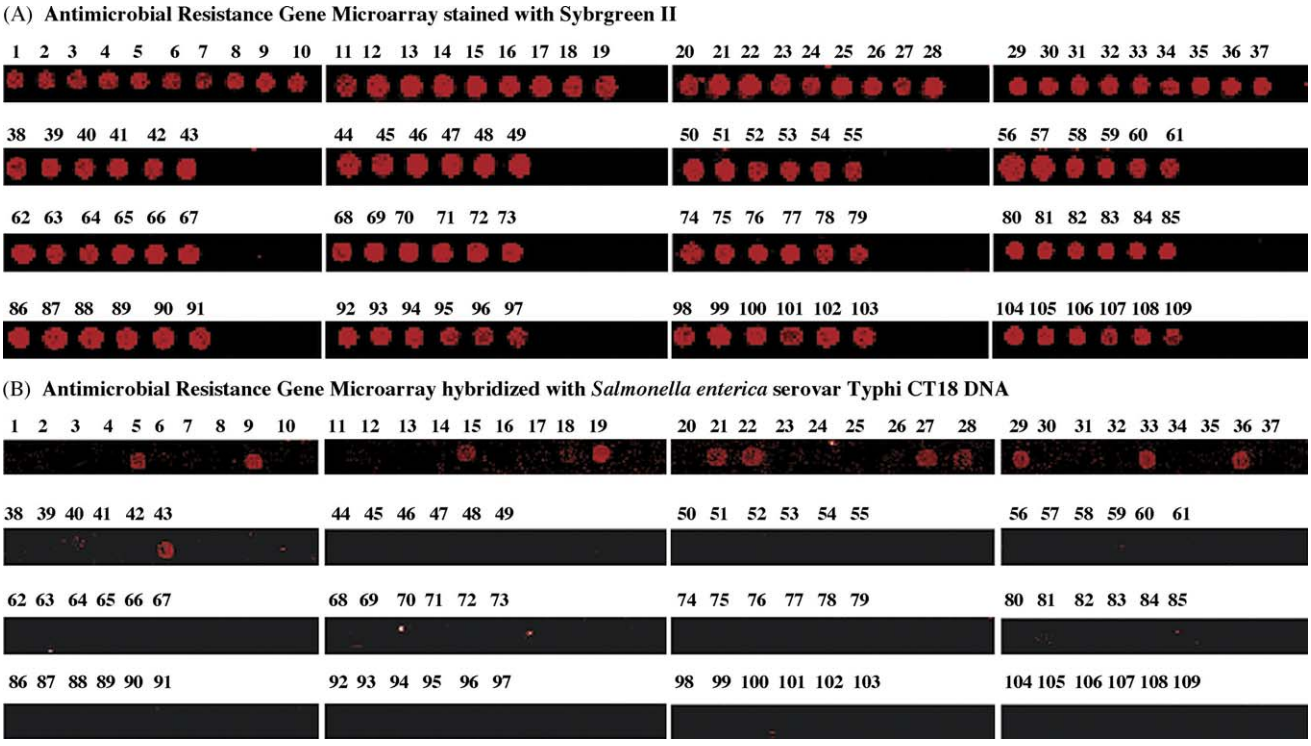


Fig. 2. Visualisation and hybridisation results for the antimicrobial resistance gene microarray. All 16 subarrays of the antimicrobial resistance gene microarray are shown. (A) Detection of oligonucleotide probe spots by Sybrgreen II® staining. The number assigned to the oligonucleotide on the microarray corresponds to its target gene listed in Table 1. (B) Hybridisation results for *Salmonella enterica* serovar Typhi CT18 DNA. Hybridisations were scored positive for the following antimicrobial resistance genes and transfer elements: 5 *aph6* (*strB*), 9 *dfrA1*, 15 *aph3*" (*strA*)*, 19 *tnpA*, 21 bla_{TEM}, 22 *intI1**, 27 *tnpM*, 28 *aph3*" (*strA*)*, 29 *aadA1*, 33 *sulII*, 36 *intI1** and 43 *cat4* (* indicates duplicate probes).

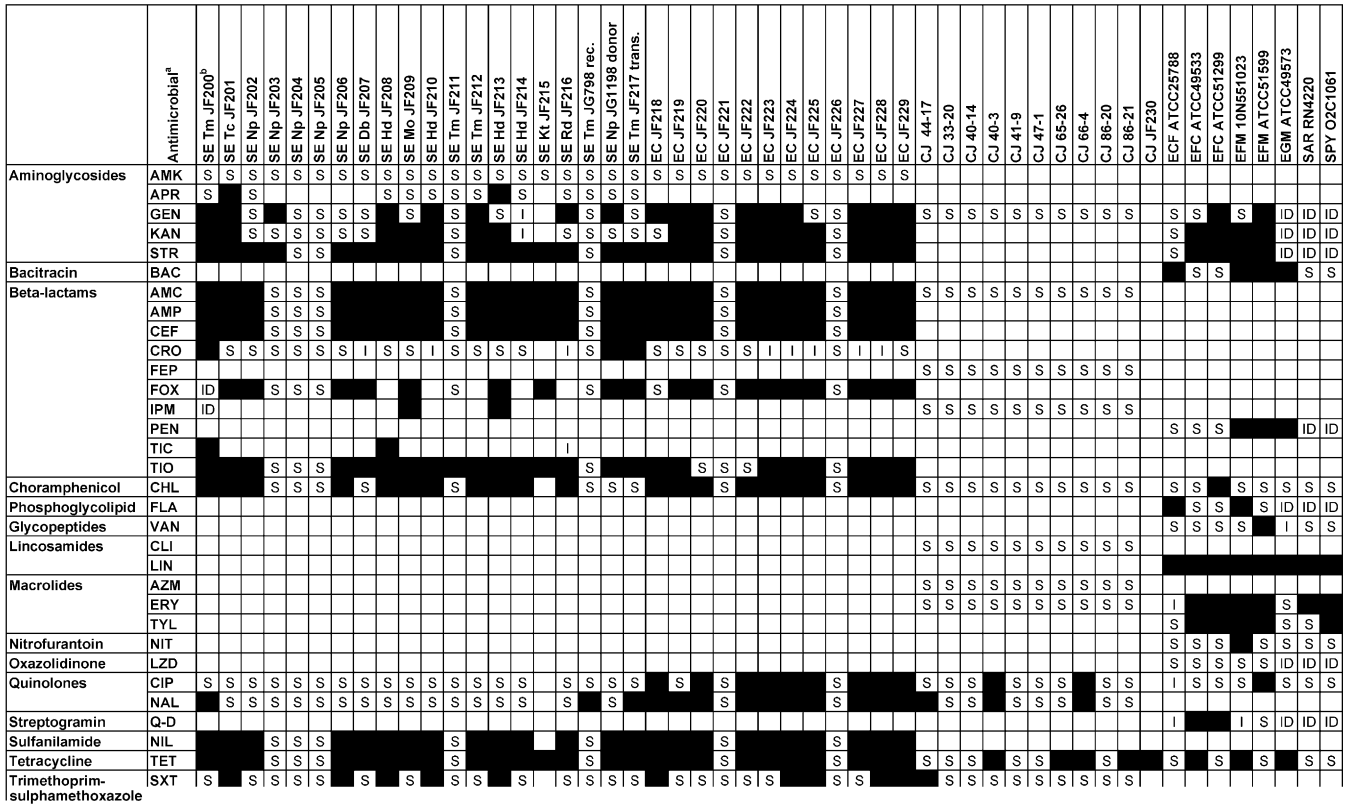
(less than 15 contiguous identical nucleotides and 43/70 nt over the length of the probe). The identical probes (denoted with a *) exhibited equivalent hybridisations and resulted in the same absence or presence call for their genes, verifying the consistency of probe synthesis, microarray construction and hybridisation across the surface of the slide (data not shown). For the remainder of the isolates analysed in this study, Cy5-labelled LT2 was included in all hybridisations for quality control, and genes were scored positive if the hybridisation was above the 2000 IU threshold. Genes detected by the microarray are indicated in Table 2 by a filled block.

3.3. Antimicrobial resistance gene microarray analysis of test isolates

The ability of the microarray to detect resistance genes in diverse bacterial strains was tested by hybridising DNA from a variety of resistant and sensitive isolates with unknown gene content. These bacteria and their phenotypic antimicrobial resistance patterns determined by growth in the presence of the antimicrobial are shown in Table 3, whilst hybridisation results are shown in Table 2. Hybridisation results from a range of *S. enterica* serotypes correlated well with the resistance patterns observed in these isolates. For example, MDR strain SE Tc JF201 was found to have 16 antimicrobial resistance genes encoding resistance for aminoglycosides, chloramphenicol, β-lactams, trimethoprim, sulfonomides and tetracycline (Table 2). These results are in agreement with the observed phenotype of this strain, which was resistant to all antimicrobials tested except the fluoroquinolones (Table 3). As expected, the hybridisation of antimicrobial-sensitive isolates SE Np JF204 and SE Np JF205 did not detect any antimicrobial resistance genes in these strains. Similarly, for most other isolates tested, the resistance genes detected by the microarray are consistent with the phenotype determined by growth with the corresponding antimicrobial compound. There were some exceptions where resistance genes were detected by the microarray but not detected by phenotype, such as the *aacC1* gene (encoding gentamicin resistance) in SE Mo JF209. These results were expected, since genes detected by hybridisation may not be functional or expressed during phenotypic tests. Conversely, some isolates were resistant to antimicrobial compounds but no genes were detected by hybridisation, likely due to the lack of a probe on the test microarray. An example of this is SE Rd JF216 that was resistant to gentamicin but had no gene detected.

The microarray was also tested for its ability to detect genes in *E. coli*, which is a commensal organism as well as a primary pathogen and is a potential reservoir/donor for antimicrobial resistance and MDR genes on plasmids, phage and transposons [43]. Twelve *E. coli* strains were hybridised to the microarray. The labelled DNA for all strains hybridised

Table 3
Antimicrobial susceptibility phenotypes of isolates used in this study



Clinical and Laboratory Standards Institute (CLSI) breakpoints were used to determine resistance phenotype. Filled block (■), resistant; S, susceptible; I, intermediate; ID, indeterminate (due to lack of CLSI standard); open block (□), not assayed.

[a]Antimicrobials: AMK, amikacin; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; APR, apramycin; FOX, cefoxitin; TIO, ceftiofur; CRO, ceftriaxone; CEF, cefalothin; CHL, chloramphenicol; CIP, ciprofloxacin; IPM, imipenem; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; NIL, sulfanilamide; TET, tetracycline; TIC, ticarcillin; SXT, trimethoprim–sulphamethoxazole; AZM, azithromycin; CLI, clindamycin; ERY, erythromycin; FEP, cefepime; BAC, bacitracin; FLA, flavomycin; LIN, lincomycin; LZD, linezolid; NIT, nitrofurantoin; PEN, penicillin; Q-D, quinupristin/dalfopristin (Synercid); TYL, tylosin; VAN, vancomycin.

[b]Isolates: SE, *Salmonella enterica*; Tm, serovar Typhimurium; Tc, serovar Typhimurium variant Copenhagen; Np, serovar Newport; Mo, serovar Montevideo; Ty, serovar Typhi; Db, serovar Derby; Hd, serovar Heidelberg; Kt, serovar Kentucky; Rd, serovar Reading; EC, *Escherichia coli*; CJ, *Campylobacter jejuni*; ECF, *Enterococcus casseliflavus*; EFC, *Enterococcus faecalis*; EFM, *Enterococcus faecium*; EGM, *Enterococcus gallinarum*; SAR, *Staphylococcus aureus*; SPY, *Streptococcus pyogenes*. Isolates used for the conjugation: recipient (SE Tm JG798 rec.), donor (SE Np JG1198 donor) and transconjugant (SE Tm JF217 trans.) are indicated.

to the PCR probe for the 23S ribosomal RNA gene (a universal positive control for bacterial DNA) as well as to ca. 70% of the other *Salmonella* genes shared by *E. coli*. The antimicrobial resistance gene oligonucleotide probes detected more than a dozen antimicrobial resistance genes and mobile elements in each of the eight MDR *E. coli* strains assayed; many of these genes were common to those found in the *S. enterica* MDR strains (Table 2). The 70-mer probes also performed as expected with sensitive strains EC JF221 and EC JF226 (Table 3), having very few hybridisations to antimicrobial resistance gene probes (Table 2). Similar to the *Salmonella* strains, the *E. coli* results correlated well with the resistance patterns determined by phenotypic analysis (Table 3).

The microarray was tested for its ability to detect antimicrobial resistance genes in *Campylobacter*. Labelled *C. jejuni* DNA hybridised only to the *Salmonella* PCR probe for the 23S ribosomal RNA gene (data not shown). As expected, no other PCR probes showed positive hybridisations owing to the significant sequence divergence in genes common to *Salmonella* and *Campylobacter*. All but one of the *C. jejuni* strains tested hybridised to the oligonucleotide probes for the *cmeABCR* genes, and all strains resistant to tetracycline (Table 3) hybridised to the *tet*(O) probe (Table 2) [37,44–46]. The *aphA-3* gene was identified in one isolate. This gene has previously been found in *Campylobacter* and is similar in sequence to the *Enterococcus* spp. *aphA-3* gene that the probe was designed to detect [47]. This high similarity in sequence could explain the hybridisation of this strain with the *aphA-3* antimicrobial resistance gene probe. As with the other Gram-negative bacteria, the resistance phenotypes of the tested strains were consistent with the genes detected (Tables 2 and 3).

Gram-positive bacteria are also known to act as reservoirs of antimicrobial resistance genes, plasmids and transposons. *Enterococcus*, *Staphylococcus* and *Streptococcus* spp. were used as Gram-positive representatives for hybridisation to the microarray. None of the Gram-positive strains tested hybridised with any *Salmonella* gene PCR product probes except the 23S ribosomal RNA gene positive control. These Gram-positive bacteria were positive control strains for the PCR analysis of specific antimicrobial resistance genes [31,32,48]. These genes were detected by the microarray and included *aphA-3* in EFM 10N551023, *aadE* in EFC ATCC49533, *vanC* in EFC ATCC25788, *erm*(C) in SAR RN4220, *erm*(B) in SPY O2C1061, *vanC* in EGM ATCC49573, *vanB2* in EFC ATCC51299 and *vanA* in EFM ATCC51599. Additional antimicrobial resistance genes were also detected by microarray analysis and were generally consistent with the resistance phenotypes of these bacteria (Tables 2 and 3).

3.4. Antimicrobial resistance gene microarray analysis of gene transfer by conjugation

Antimicrobial-resistant bacteria can harbour antimicrobial resistance genes on multiple plasmids, some of which can be transferred from one bacterium to another [4,30,49,50]. Transfer by conjugation can be assayed for by selection for the resistance phenotype after mating. Owing to the possibility of resistance genes on multiple plasmids, transconjugants require thorough genetic screening to determine which genes were transferred with a plasmid. To establish the ability of the microarray to do this, it was used to monitor the in vitro transfer of antimicrobial resistance genes on a MDR plasmid from a donor strain (SE Np JG1198 donor) to a recipient strain (SE Tm JG798 rec.) by conjugation. Transfer of a plasmid encoding the $bla_{\text{CMY-2}}$ gene was confirmed by Southern blot detection [51] of the plasmid in the transconjugant (data not shown). Microarray hybridisations detected eight antimicrobial resistance genes including $bla_{\text{CMY-2}}$, *dfrA1*, *aph3*" (*strA*), *aph6* (*strB*), *sulII*, *tet*(A), *tet*(R) and *tnpA* in both the donor (SE Np JG1198 donor) and the transconjugant (SE Tm JF217 trans.), but none in the recipient (SE Tm JG798 rec.) (Table 2). Sensititer analysis verified identical resistance patterns in the donor (SE Np JG1198 donor) and the transconjugant (SE Tm JF217 trans.) except for nalidixic acid, used for counterselection in the recipient (SE Tm JG798 rec.), and gentamicin in the donor (SE JG1198 trans.) (Table 3). Because no gentamicin resistance gene was detected by the microarray, it was not determined whether this gene was transferred to the recipient strain.

3.5. Verification of antimicrobial resistance gene microarray results by alternative methods

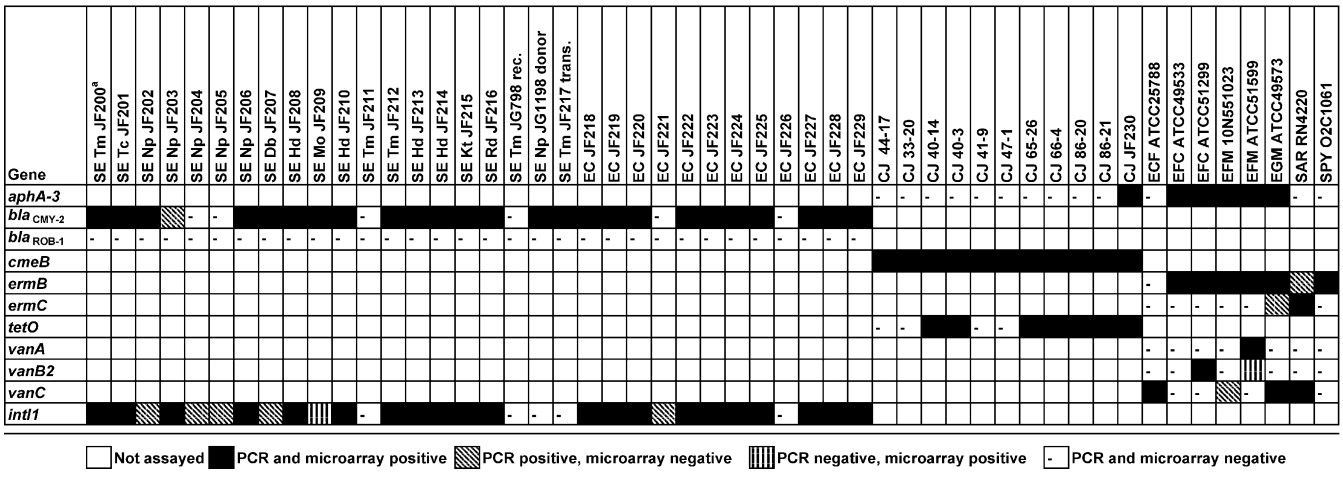
Of all the strains tested, only two (the fully sequenced *Salmonella* LT2 and CT18) were highly defined for antimicrobial resistance gene content. Several of the strains were known to harbour one specific gene but demonstrated hybridisations to multiple antimicrobial resistance gene probes (Table 2). To confirm hybridisation results, 11 genes were chosen for detection by PCR. The genes and primer sequences used in PCR confirmation are shown in Table 4 and the strains tested and their PCR results are listed in Table 5. The majority of PCR results (166/177) were consistent with the microarray data, and positive controls for each PCR reaction yielded amplification products of the correct size (data not shown). However, PCR and microarray hybridisation data differed for genes in 11 of 177 isolates as indicated by hatched boxes in Table 5. Nine of these were PCR positive and microarray negative, for example SE Np JF203, which was PCR positive for the $bla_{\text{CMY-2}}$ gene but negative by hybridisation to the $bla_{\text{CMY-2}}$ probe on the microarray. Conversely, two were PCR negative and microarray positive, for example EFM ATCC51599 hybridised to the *vanB2* probe on the microarray but was negative by PCR analysis.

Another method for analysis of antimicrobial resistance genes is detection of the gene by Southern blot hybridisation. This technique requires many steps but has the advantage that it may also identify the genetic element on which the target gene resides. This approach was used to determine the presence of the $bla_{\text{CMY-2}}$ gene in the *S. enterica* isolates SE Np JG1198, SE Tm JG798, SE Tm JF217, SE Tc JF201, SE Tm JF212, SE Hd JF214 and SE Rd JF216. The $bla_{\text{CMY-2}}$ gene was detected in all strains except for the negative control, SE Tm JG798 [51]. The gene was located on plasmids in these

Table 4
Polymerase chain reaction primers used to confirm the status of antimicrobial resistance genes

Gene	Forward primer	Reverse primer	Reference
aphA-3	CTGATCGAAAAATACCGCT	ACAATCCGATATGTCGATGGAG	[17]
$bla_{\text{CMY-2}}$	GACAGCCTCTTTCTCCACA	TGGACGAAGGCTACGTA	[3]
$bla_{\text{ROB-1}}$	TGTTTGCAATCGCTGCC	TTATCGTACACTTTCCA	[13]
cmeB	GACGTAATGAAGGAGAGCCA	CTGATCCACTCCAAGCTATG	[41]
erm(B)	TAACGACGAAACTGGCTAAAAT	ATCTGTGGTATGGCGGGTAAG	[44]
erm(C)	AGTACAGAGGTGTAATTTCG	AATTCCTGCATGTTTTAAGG	[44]
tet(O)	TAATGAAGATTCCGACAATT	CGGCAACAGTATTTCGTT	[18]
vanA	CATGAATAGAATAAAAGTTGCAATA	CCCCTTTAACGCTAATACGATCAA	[26]
vanB2	AAGCTATGCAAGAAGCCATG	CCGACAATCAAATCATCCTC	[26]
vanC	CGGGGAAGATGGCAGTAT	CGCAGGGACGGTGATTTT	[35]
intI1	ACATGTGATGGCGACGCACGA	ATTTCTGTCCTGGCTGGCGA	[13]

Table 5
Correlation between microarray data and polymerase chain reaction (PCR)



[a] Isolates: SE, *Salmonella enterica*; Tm, serovar Typhimurium; Tc, serovar Typhimurium variant Copenhagen; Np, serovar Newport; Mo, serovar Montevideo; Ty, serovar Typhi; Db, serovar Derby; Hd, serovar Heidelberg; Kt, serovar Kentucky; Rd, serovar Reading; EC, *Escherichia coli*; CJ, *Campylobacter jejuni*; ECF, *Enterococcus casseliflavus*; EFC, *Enterococcus faecalis*; EFM, *Enterococcus faecium*; EGM, *Enterococcus gallinarum*; SAR, *Staphylococcus aureus*; SPY, *Streptococcus pyogenes*. Isolates used for the conjugation: recipient (SE Tm JG798 rec.), donor (SE Np JG1198 donor) and transconjugant (SE Tm JF217 trans.) are indicated.

strains that varied in size from ca. 150 Kbp to 200 Kbp (data not shown).

3.6. Assessment of the antimicrobial resistance gene microarray performance

The test microarray detected 61 resistance genes in a variety of bacteria. Confirmation of the hybridisation data by duplicate probes, phenotypic tests, PCR assays and Southern blot analysis indicated that the 70-mer oligonucleotide microarray performed well. As with other microarrays designed to detect antimicrobial resistance genes, some probes did not hybridise during testing [15,16]. Thirty probes did not hybridise with any of the strains tested, however none of the strains were known to contain the genes these probes were designed to detect. Interpretation of these negative hybridisation results as absent genes will require positive controls, therefore these genes are not listed in Table 2. This is an important consideration because a microarray designed to detect all sequenced antimicrobial resistance genes will contain many hundreds of probes some of which may lack positive controls. Consequently, negative hybridisations for probes without positive controls should be interpreted as not detected rather than absent.

Three probes also gave aberrant results. The *vanD* and *vanH* probes hybridised to several *Salmonella* and *E. coli* strains. These genes confer vancomycin resistance and are found in *Enterococcus* spp. and other Gram-positive bacteria. Analysis of the probe sequences by BLAST against the *Salmonella* and *E. coli* genomes revealed regions of identity to several ATP-binding components of transport systems in these Gram-negative bacteria (data not shown). Similarly, the probe for *msr*(A) hybridised to all *E. coli* and most *Salmonella* isolates. Investigation of the sequence used to design the probe found that the wrong gene had been selected for probe design. Rather than a multidrug efflux pump, the gene for methionine sulfoxide reductase from *E. coli* was selected. Data from these three probes were disregarded and are not reported in Table 2.

4. Discussion

Identification of the antimicrobial resistance genes responsible for resistant phenotypes is made difficult by the requirement of multiple assays for each gene or a group of genes [7]. DNA microarray techniques have recently been described for the simultaneous detection of multiple antimicrobial resistance genes in MDR isolates [9,10,15,16]. In experiments presented here, we demonstrate that a simple approach to DNA microarray development can produce a practical tool for the detection of multiple antimicrobial resistance genes in a variety of diverse bacteria. The techniques described in this report offer several advantages over traditional PCR product microarray construction and demonstrate a simple approach to produce a working microarray with oligonucleotides. The target genes were selected from the literature and sequences were obtained from the NCBI database. Simple programs such as MELTING [20] and BLAT [21] were employed for probe design and to determine specificity. Synthetic oligonucleotide probes eliminated the requirement for template, PCR primers, PCR reactions, scoring and clean-up prior to array construction [12]. While this microarray was being evaluated, a number of oligoarray design tools were described [52–54] and could be used to facilitate further the design of oligonucleotide probes for microarray construction. More

importantly, owing to the continual decline in synthesis fees, the microarray can easily be expanded by the addition of oligonucleotide probes to detect thousands more antimicrobial resistance, virulence, plasmid, transposon, integron and phage genes.

In the present study, 70-mer oligonucleotide probes performed as well as PCR product probes on the microarray. Although extra steps were taken in designing the probes (i.e. the addition of spacers and amino linkers), it was found that these were not necessary [55]. The oligonucleotides functioned well when treated in the same manner used for PCR products and both were compatible on the same array, as seen in Fig. 1. However, some unique features of oligonucleotide probes were identified during this study. The 70-mer oligonucleotide probes demonstrated higher specificity for their target genes than the whole open reading frame PCR products. It has previously been shown that PCR products can report on genes that are divergent in sequence, whilst oligonucleotides can only accommodate a few mismatches within the probe sequence [22]. For example, the *tet*(A) and *tet*(R) probes could only detect certain alleles of those genes. To detect all members of a gene family, a probe would need to be designed to hybridise to a region of conserved sequence in the alleles, or a separate probe would need to be designed to detect each allele individually. Conversely, this high specificity offers the advantage of discrimination between individual members within gene families. Carefully designed probes would allow the investigation of the evolution of gene families and the epidemiology of divergent resistance genes. On the other hand, long oligonucleotides (50–70-mer) cannot discriminate between the single nucleotide polymorphisms (SNPs) used to classify groups of genes with very small changes. Shorter probes (i.e. 17–27-mer) and amplification of the target sequence have been used for SNP analysis by microarrays, and have been recently described for the detection of the TEM β-lactamases by Grimm et al. [56]. Therefore, microarray probes afford a continuum of utilities such as PCR probe detection of moderately divergent genes, long oligonucelotide probe detection of sequences that contain a few mismatches, and short probe detection of individual SNPs. The test microarray presented in this report offers a compromise between these extremes.

Another advantage of DNA microarray detection of antimicrobial resistance genes is that this technique works on any bacteria from which DNA can be extracted. For instance, MDR *S. enterica* isolates representing a variety of serotypes hybridised to antimicrobial resistance genes previously found in *S. enterica* serovar Typhimurium DT 104 and *S. enterica* serovar Newport [57,58]. The widespread nature of these antimicrobial resistance genes among MDR *Salmonella* has previously been reported and the array data presented in the present study confirm those observations. Interestingly, many of these genes were also found in *E. coli* MDR strains, suggesting the exchange of antimicrobial resistance genes between these closely related bacteria or some other common origin [30,43]. The possibility of transmission of antimicrobial resistance genes between non-related bacteria was demonstrated by the detection of the *aphA-3* gene in *C. jejuni* as well as in *Enterococcus* spp. [47]. Additionally, the conjugation experiments demonstrate that the exchange of multiple genetic markers can be observed in vitro by microarray. This provides a new tool for the study of these events in environmental or in vivo animal models. As the numbers of target genes can be expanded, the microarray can also be used to observe the horizontal transfer of other genetic elements not associated with antimicrobial resistance.

We observed occasional discrepancies between microarray results and those of the other methods used (PCR, Southern hybridisation and phenotypic testing). For example, EFC ATCC51299 was sensitive to vancomycin (Table 3) but microarray and PCR analysis each detected the *vanB2* gene (Tables 2 and 5) [38,48]. There are several possible explanations for these results. Some genes may not hybridise to the microarray owing to divergence within the 70 bp region of the oligonucleotide probe. It is equally likely that failure of PCR analysis to detect a gene that hybridised to the microarray could be due to divergence within the region to which the PCR primers were designed to anneal. The variability of results from target genes is exemplified by *intI1*, which was highly variable, resulting in PCR and microarray analyses that were different for 6 of 30 isolates assayed. BLAST analysis showed that the PCR primers would amplify *intI1* genes and many related genes found in the database, whereas the probe would only detect a specific subset of *intI1* genes. These types of results demonstrate different aspects of the genes under investigation and show how these techniques could be used to complement each other. Some researchers have already suggested that genetic tests should be used for identification of resistance genes when they cannot be easily detected by standard phenotypic tests, or require complex, slow or difficult to interpret tests [7].

In conclusion, this oligonucleotide microarray was easily, simply and inexpensively constructed, and is capable of identifying a variety of antimicrobial resistance genes in diverse bacteria. The methods described in this study can be used to design a microarray capable of detecting all sequenced antimicrobial resistance genes. So far we have identified 681 more resistance genes in the NCBI database (unpublished data) and will add probes for their detection to the microarray. Whilst microarray analysis is not as cheap as PCR detection for an individual gene, microarrays can assay for many genes simultaneously and the method is thus outstanding value on a per gene basis. Microarrays are currently limited by the number of isolates they can analyse at one time. However, the data collected from these studies can be used to develop techniques for rapid detection of antimicrobial resistance genes and screening of large numbers of clinical isolates. Data from the oligonucleotide microarrays can be adapted to automated, rapid, high throughput technologies (i.e. Q-PCR, hybridisation beads, flow cytometry microarrays or quantum dot detected hybridisation) capable of analysing hundreds of samples at once.

Acknowledgments

The authors thank Rosie Minish, Jennifer Garland, Russ Turpin, Jovita Haro, Lari Hiott, Takiyah Ball and Jeanetta Tankson for technical assistance. We also thank Anne Summers for critical reading of the manuscript. M.M. was funded by NIH grants R01AI34829 and R21 AI057733 and by the generosity of Sidney Kimmel.

References

[1] Salyers AA, Shoemaker NB. Resistance gene transfer in anaerobes: new insights, new problems. Clin Infect Dis 1996;23:S36–43.
[2] Powers JH. Antimicrobial drug development—the past, the present, and the future. Clin Microbiol Infect 2004;10(Suppl. 4):23–31.
[3] Martinez JL, Baquero F. Mutation frequencies and antibiotic resistance. Antimicrob Agents Chemother 2000;44:1771–7.
[4] Gebreyes WA, Altier C. Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. enterica serovar Typhimurium isolates from swine. J Clin Microbiol 2002;40:2813–22.
[5] National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Wayne, PA: NCCLS; 2002.
[6] National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Wayne, PA: NCCLS; 2003.
[7] Cockerill III FR. Genetic methods for assessing antimicrobial resistance. Antimicrob Agents Chemother 1999;43:199–212.
[8] Shalon D, Smith SJ, Brown PO. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res 1996;6:639–45.
[9] Call DR, Bakko MK, Krug MJ, Roberts MC. Identifying antimicrobial resistance genes with DNA microarrays. Antimicrob Agents Chemother 2003;47:3290–5.
[10] Volokhov D, Chizhikov V, Chumakov K, Rasooly A. Microarray analysis of erythromycin resistance determinants. J Appl Microbiol 2003;95:787–98.
[11] Fukiya S, Mizoguchi H, Tobe T, Mori H. Extensive genomic diversity in pathogenic *Escherichia coli* and *Shigella* strains revealed by comparative genomic hybridization microarray. J Bacteriol 2004;186:3911–21.
[12] Carvalho B, Ouwerkerk E, Meijer GA, Ylstra B. High resolution microarray comparative genomic hybridisation analysis using spotted oligonucleotides. J Clin Pathol 2004;57:644–6.
[13] Taboada EN, Acedillo RR, Carrillo CD, et al. Large-scale comparative genomics meta-analysis of *Campylobacter jejuni* isolates reveals low level of genome plasticity. J Clin Microbiol 2004;42:4566–76.
[14] Wang J, Meza-Zepeda LA, Kresse SH, Myklebost O. M-CGH: analysing microarray-based CGH experiments. BMC Bioinformatics 2004;5:74.
[15] Perreten V, Vorlet-Fawer L, Slickers P, Ehricht R, Kuhnert P, Frey J. Microarray-based detection of 90 antibiotic resistance genes of gram-positive bacteria. J Clin Microbiol 2005;43:2291–302.
[16] van Hoek AH, Scholtens IM, Cloeckaert A, Aarts HJ. Detection of antibiotic resistance genes in different *Salmonella* serovars by oligonucleotide microarray analysis. J Microbiol Methods 2005;62:13–23.
[17] Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 2001;65:232–60.
[18] Rasheed JK, Tenover FC. Clinical microbiology. 8th ed. Washington, DC: ASM Press; 2003.
[19] Vanhoof R, Hannecart-Pokorni E, Content J. Nomenclature of genes encoding aminoglycoside-modifying enzymes. Antimicrob Agents Chemother 1998;42:483.
[20] Le Novere N. MELTING, computing the melting temperature of nucleic acid duplex. Bioinformatics 2001;17:1226–7.
[21] Kent WJ. BLAT—the BLAST-like alignment tool. Genome Res 2002;12:656–64.
[22] Porwollik S, Frye J, Florea LD, Blackmer F, McClelland M. A non-redundant microarray of genes for two related bacteria. Nucleic Acids Res 2003;31:1869–76.
[23] Porwollik S, Boyd EF, Choy C, et al. Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. J Bacteriol 2004;186:5883–98.
[24] McClelland M, Sanderson KE, Spieth J, et al. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 2001;413:852–6.
[25] Parkhill J, Dougan G, James KD, et al. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature 2001;413:848–52.
[26] Jackson CR, Fedorka-Cray PJ, Barrett JB, Ladely SR. Genetic relatedness of high-level aminoglycoside-resistant enterococci isolated from poultry carcasses. Avian Dis 2004;48:100–7.
[27] Chesneau O, Lailler R, Derbise A, El Solh N. Transposition of IS1181 in the genomes of *Staphylococcus* and *Listeria*. FEMS Microbiol Lett 1999;177:93–100.
[28] Clewell DB, Franke AE. Characterization of a plasmid determining resistance to erythromycin, lincomycin, and vernamycin Balpha in a strain *Streptococcus pyogenes*. Antimicrob Agents Chemother 1974;5:534–7.
[29] Porwollik S, Wong RM, Sims SH, Schaaper RM, DeMarini DM, McClelland M. The DeltauvrB mutations in the Ames strains of *Salmonella* span 15 to 119 genes. Mutat Res 2001;483:1–11. [Erratum. Mutat Res 2001;484:107–10.]
[30] Allen KJ, Poppe C. Occurrence and characterization of resistance to extended-spectrum cephalosporins mediated by beta-lactamase CMY-2 in *Salmonella* isolated from food-producing animals in Canada. Can J Vet Res 2002;66:137–44.
[31] Khan SA, Nawaz MS, Khan AA, Hopper SL, Jones RA, Cerniglia CE. Molecular characterization of multidrug-resistant *Enterococcus* spp. from poultry and dairy farms: detection of virulence and vancomycin resistance gene markers by PCR. Mol Cell Probes 2005;19:27–34.
[32] Patel R, Uhl JR, Kohner P, Hopkins MK, Cockerill III FR. Multiplex PCR detection of *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* genes in enterococci. J Clin Microbiol 1997;35:703–7.
[33] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. New York, NY: Cold Spring Harbor Press; 1989.
[34] Wain J, Diem Nga LT, Kidgell C, et al. Molecular analysis of incHI1 antimicrobial resistance plasmids from *Salmonella serovar* Typhi strains associated with typhoid fever. Antimicrob Agents Chemother 2003;47:2732–9.
[35] Villa L, Pezzella C, Tosini F, Visca P, Petrucca A, Carattoli A. Multiple-antibiotic resistance mediated by structurally related IncL/M plasmids carrying an extended-spectrum beta-lactamase gene and a class 1 integron. Antimicrob Agents Chemother 2000;44:2911–4.
[36] Ridley A, Threlfall EJ. Molecular epidemiology of antibiotic resistance genes in multiresistant epidemic *Salmonella typhimurium* DT 104. Microb Drug Resist 1998;4:113–8.
[37] Gibreel A, Tracz DM, Nonaka L, Ngo TM, Connell SR, Taylor DE. Incidence of antibiotic resistance in *Campylobacter jejuni* isolated in Alberta, Canada, from 1999 to 2002, with special reference to *tet*(O)-mediated tetracycline resistance. Antimicrob Agents Chemother 2004;48:3442–50.
[38] Woodford N. Epidemiology of the genetic elements responsible for acquired glycopeptide resistance in enterococci. Microb Drug Resist 2001;7:229–36.

[39] Dunny GM, Antiporta MH, Hirt H. Peptide pheromone-induced transfer of plasmid pCF10 in *Enterococcus faecalis*: probing the genetic and molecular basis for specificity of the pheromone response. Peptides 2001;22:1529–39.
[40] Roberts MC. Resistance to tetracycline, macrolide–lincosamide–streptogramin, trimethoprim, and sulfonamide drug classes. Mol Biotechnol 2002;20:261–83.
[41] Battaglia C, Salani G, Consolandi C, Bernardi LR, De Bellis G. Analysis of DNA microarrays by non-destructive fluorescent staining using SYBR green II. Biotechniques 2000;29:78–81.
[42] Wain J, Kidgell C. The emergence of multidrug resistance to antimicrobial agents for the treatment of typhoid fever. Trans R Soc Trop Med Hyg 2004;98:423–30.
[43] Charpentier E, Gerbaud G, Courvalin P. Conjugative mobilization of the rolling-circle plasmid pIP823 from *Listeria monocytogenes* BM4293 among gram-positive and gram-negative bacteria. J Bacteriol 1999;181:3368–74.
[44] Pumbwe L, Randall LP, Woodward MJ, Piddock LJ. Expression of the efflux pump genes *cmeB*, *cmeF* and the porin gene *porA* in multiple-antibiotic-resistant *Campylobacter jejuni*. J Antimicrob Chemother 2004;54:341–7.
[45] Randall LP, Ridley AM, Cooles SW, et al. Prevalence of multiple antibiotic resistance in 443 *Campylobacter* spp. isolated from humans and animals. J Antimicrob Chemother 2003;52:507–10.
[46] Lin J, Michel LO, Zhang Q. CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. Antimicrob Agents Chemother 2002;46:2124–31.
[47] Gibreel A, Skold O, Taylor DE. Characterization of plasmid-mediated *aphA-3* kanamycin resistance in *Campylobacter jejuni*. Microb Drug Resist 2004;10:98–105.
[48] Hanaki H, Yamaguchi Y, Nomura S, Nagayama A, Sunakawa K. Rapid detection and differentiation method of VanA, VanB and VanC phenotypes in vancomycin-resistant enterococci. Int J Antimicrob Agents 2004;23:502–5.
[49] Ahmer BM, Tran M, Heffron F. The virulence plasmid of *Salmonella typhimurium* is self-transmissible. J Bacteriol 1999;181:1364–8.
[50] Clewell DB, editor. Bacterial conjugation. 1st ed. New York, NY: Plenum Press; 1993.
[51] Gray JT, Hungerford LL, Fedorka-Cray PJ, Headrick ML. Extended-spectrum-cephalosporin resistance in *Salmonella enterica* isolates of animal origin. Antimicrob Agents Chemother 2004;48:3179–81.
[52] Chou HH, Hsia AP, Mooney DL, Schnable PS. Picky: oligo microarray design for large genomes. Bioinformatics 2004;20:2893–902.
[53] Gordon PM, Sensen CW. Osprey: a comprehensive tool employing novel methods for the design of oligonucleotides for DNA sequencing and microarrays. Nucleic Acids Res 2004;32:e133.
[54] Rahmann S. Fast large scale oligonucleotide selection using the longest common factor approach. J Bioinform Comput Biol 2003;1:343–61.
[55] Lyons P. Advances in spotted microarray resources for expression profiling. Brief Funct Genomic Proteomic 2003;2:21–30.
[56] Grimm V, Ezaki S, Susa M, Knabbe C, Schmid RD, Bachmann TT. Use of DNA microarrays for rapid genotyping of TEM beta-lactamases that confer resistance. J Clin Microbiol 2004;42:3766–74.
[57] Boyd D, Peters GA, Cloeckaert A, et al. Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. J Bacteriol 2001;183:5725–32.
[58] Khan AA, Nawaz MS, Khan SA, Cerniglia CE. Detection of multidrug-resistant *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. FEMS Microbiol Lett 2000;182:355–60.